NifB-dependent *in vitro* synthesis of the iron–molybdenum cofactor of nitrogenase

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Biological nitrogen fixation, an essential process of the biogeochemical nitrogen cycle that supports life on Earth, is catalyzed by the nitrogenase enzyme. The nitrogenase active site contains an iron and molybdenum cofactor (FeMo-co) composed of 7Fe-9S-Mo-homocitrate and one not-yet-identified atom, which probably is the most complex [Fe–S] cluster in nature. Here, we show the *in vitro* synthesis of FeMo-co from its simple constituents, Fe, S, Mo, and homocitrate. The *in vitro* FeMo-co synthesis requires purified NifB and depends on *S*-adenosylmethionine, indicating that radical chemistry is required during FeMo-co assembly.

iron–sulfur | *S*-adenosylmethionine radical

Biological nitrogen fixation, the conversion of atmospheric N_2 to ammonium, is an essential process of the biogeochemical nitrogen cycle that supports life on Earth (1). The major part of biological nitrogen fixation is catalyzed by the molybdenum nitrogenase, although some nitrogen-fixing bacteria additionally contain alternative vanadium or iron-only nitrogenases that are expressed when molybdenum is not available (2). The molybdenum nitrogenase carries at its active site the most complex metallocluster known in biology: the iron and molybdenum cofactor (FeMo-co), composed of seven Fe, nine S, one Mo, one homocitrate, and one atom of unidentified nature (3, 4). A number of nitrogen fixation (*nif*) genes have been shown to be involved in FeMo-co biosynthesis and the formation of an active nitrogenase enzyme (5, 6). Among these, the *nifB* gene has been long recognized as crucial for nitrogen fixation because *nifB* participates in an early synthetic step that is common to the biosyntheses of FeMo-co and the analogous iron-vanadium (FeV) and iron-only (FeFe) cofactors, carried by the vanadium and the iron-only nitrogenases, respectively (7). Here, we show the purification of a functional NifB, which has allowed us to perform *in vitro* synthesis of FeMo-co from its simple constituents, Fe, S, Mo, and homocitrate. The metabolic product of NifB, termed NifB-co, had been isolated during early NifB purification attempts and was shown to be some type of [Fe–S] cluster that serves as a precursor for *in vitro* FeMo-co synthesis (8). However, until now, it has not been possible to synthesize either NifB-co or FeMo-co from their simplest components. This synthesis is achieved here through the purification of NifB and the development of a NifB activity assay that drives the synthesis of FeMo-co and the formation of active nitrogenase, which entails definitive progress in the field of nitrogen fixation and in the study of complex metallocluster biosynthesis in general.

Results and Discussion

The *Azotobacter vinelandii nifB* gene was shuffled from its wild-type position to the *nifH* locus in the chromosome forming a chimeric operon with the structural nitrogenase genes (Fig. 4, which is published as supporting information on the PNAS web site). The resulting strain, UW232, expresses a His-10-NifB variant at levels that are 5-fold higher than the wild-type strain while conserving the regulation of its expression by ammonium (Fig. 4). Strain UW232 is able to fix N_2 and grow diazotrophically, indicating that His-10-NifB is functional *in vivo* (Fig. 4). Hereafter, we refer to the His-10-NifB variant simply as NifB. NifB was purified under rigorous anaerobic conditions from UW232 cells (Fig. 1*A*). The average NifB purification yield is 20 mg·kg^{-1} of cells with an enrichment of 2,400-fold. NifB preparations are yellow/brown and displayed a positive signal for Fe when subjected to anoxic native PAGE and stained for the metal (Fig. 1*B*). Analytical gel filtration and Fe analysis show that, as isolated, NifB is a dimer of 108 ± 4 kDa that contains 11.8 ± 0.2 Fe atoms. NifB has a typical [Fe–S] protein UV-visible spectrum with a broad peak at ≈400 nm, a shoulder at 315 nm, and an A_{400}:A_{279} ratio of 0.3 (Fig. 1*C*). Exposure of NifB to air resulted in color bleaching. Addition of Fe and S under reducing conditions converted the as-isolated NifB into a form with 18 ± 0.4 Fe atoms per dimer and an A_{400}:A_{279} ratio of 0.5 (labeled as "reconstituted" in Fig. 1*C*). The A_{400}:A_{279} ratio decreases in the presence of 5 mM sodium dithionite (DTH), suggesting that DTH reduces the [Fe–S] clusters of both the as-isolated and the reconstituted NifB proteins. Amino acid sequence analysis suggests that NifB is an *S*-adenosylmethionine (SAM)-dependent enzyme, an emergent family of enzymes that display a variety of biochemical reactions but have in common the presence of an unusual [4Fe–4S] cluster coordinated by three cysteine residues and an SAM molecule (9). These proteins catalyze the reductive cleavage of SAM, producing a 5′ deoxyadenosyl radical, which initiates radical chemistry on a variety of substrates (10). NifB proteins contain the signature motif of SAM radical enzymes (CxxxCxxC) at the N-terminal region (9). NifB proteins also show the presence of nine conserved cysteine residues that could potentially coordinate additional [Fe–S] clusters (6) and a C-terminal region that is conserved among proteins able to bind FeMo-co or NifB-co (11). Interestingly, the presence of 1 mM SAM in NifB preparations altered the spectra of the reduced forms of NifB. As is the case in other SAM radical enzymes, this effect likely indicates direct interaction of SAM with an [Fe–S] cluster of NifB.

Nitrogenase enzyme is composed of dinitrogenase (NifDK or MoFe protein) and dinitrogenase reductase (NifH or Fe protein). Newly synthesized dinitrogenase is an apoenzyme that lacks FeMo-co. FeMo-co is assembled elsewhere in the cell and then is inserted into apodinitrogenase to generate an active dinitrogenase component (5). When reproducing this biosynthetic system *in vitro*, it is widely accepted that the synthesis of FeMo-co requires NifB-co, homocitrate, Mo, NifH, Mg-ATP, DTH as reductant, and the tetrameric protein NifEN, which acts as a molecular scaffold on which some of the FeMo-co assembly reactions take place (8, 12–18). NifB-co, the presumed metabolic product of NifB, is an [Fe–S] cluster of unidentified structure that serves as a precursor to FeMo-co as well as to the FeV and FeFe cofactors. NifB-co was isolated first during early NifB

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Abbreviations: FeMo-co, iron and molybdenum cofactor; DTH, sodium dithionite; SAM, *S*-adenosylmethionine; NifB-co, metabolic product of NifB; SAH, *S*-adenosylhomocysteine.

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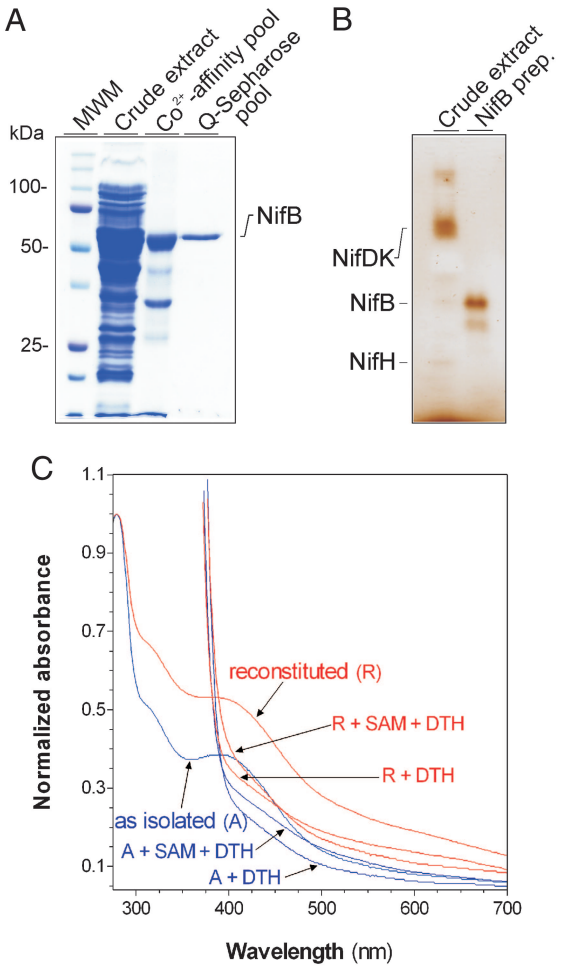

Fig. 1. Purification of NifB and UV-visible spectra of as-isolated and reconstituted NifB. (*A*) SDS/PAGE analysis of NifB purification steps (cell-free extract, 50 μg of protein; purified NifB, 2 μg of protein). (*B*) Fe staining of purified NifB after anoxic native PAGE (cell-free extract, 150 μg of protein; purified NifB, 30 μg of protein). (*C*) NifB samples (10 μM) were chemically reconstituted as described in *Materials and Methods*. Spectra were normalized to their absorbance at 279 nm.

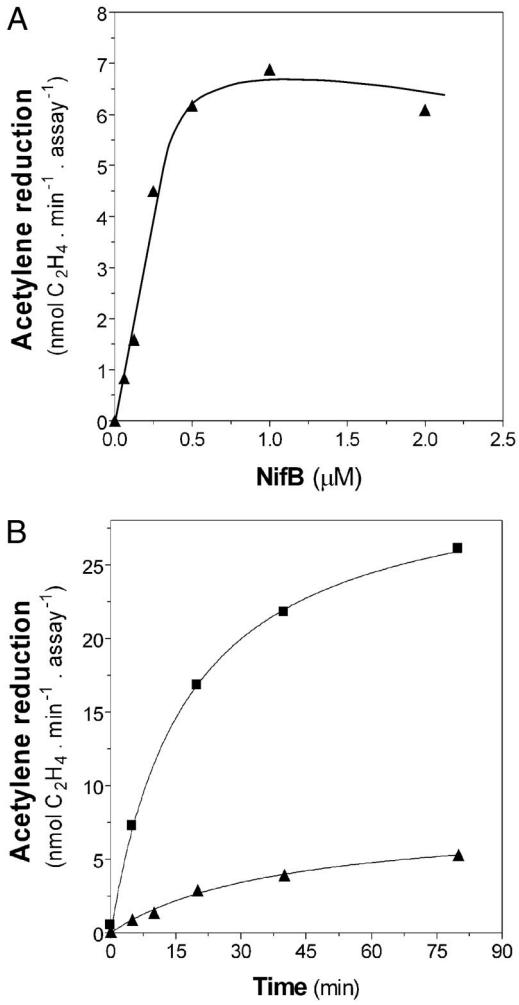

Fig. 2. NifB-dependent *in vitro* synthesis of FeMo-co. FeMo-co synthesis was determined by acetylene reduction activity of matured dinitrogenase (8). The *in vitro* FeMo-co synthesis assay is described in *Materials and Methods*. (*A*) Titration of FeMo-co synthesis assay with NifB. The indicated concentrations of NifB dimer were used. (*B*) Time course of *in vitro* FeMo-co synthesis reactions dependent on NifB (▲) or NifB-co (■). Concentrations of NifB dimer (0.48 μM) and NifB-co (8.55 μM Fe) were saturating (the number of Fe atoms per NifB-co molecule is unknown).

purification attempts, and it has been successfully used as an Fe and S donor for *in vitro* FeMo-co synthesis assays (8, 13).

The *in vitro* FeMo-co synthesis and insertion assay typically involves the addition of purified NifB-co to an extract of *A. vinelandii* UW45 (*nifB*$^-$) in the presence of an ATP-regenerating mixture, Mo, and homocitrate. Strain UW45 lacks NifB-co activity but provides the rest of the protein components required for FeMo-co synthesis and activatable apodinitrogenase. When *in vitro* FeMo-co synthesis occurs, *de novo*-synthesized FeMo-co is incorporated into apodinitrogenase to reconstitute an active dinitrogenase enzyme. The amount of activatable apodinitrogenase present in the UW45 extract is estimated in an independent reaction by adding purified FeMo-co, which is readily inserted into apodinitrogenase. The NifB-co-dependent reaction typically activates 80% of apodinitrogenase present in the extract of UW45.

As isolated, NifB was active in an *in vitro* FeMo-co synthesis assay in which 1.75 mM $(NH_4)_2Fe(SO_4)_2$, 1.75 mM Na_2S, 0.88 mM SAM, 3 mM DTH, and 0.48 μM NifB dimer substituted for the NifB-co requirement (Fig. 2*A*). Time-course comparison of NifB- and NifB-co-dependent apodinitrogenase activation shows that the NifB-co-dependent reaction resulted in higher levels of nitrogenase activity than the NifB-dependent reaction (Fig. 2*B*). Thus, it seems that the reaction catalyzed by NifB is limiting in FeMo-co synthesis *in vitro*. The saturation profile of the NifB-dependent reaction also suggests that NifB, as isolated from UW232 cells, does not carry NifB-co clusters. Adding more NifB protein to the reaction mixture does not increase FeMo-co synthesis to the levels observed on the NifB-co-dependent synthesis assay, suggesting that the NifB reaction is limited by an additional factor (or factors) in the extract. The limiting factor(s) do not seem to be required after NifB-co synthesis because the NifB-co-dependent reaction saturates at a much higher level of apodinitrogenase activation. This finding is consistent with previous observations that FeMo-co precursors (other than homocitrate) are not accumulated when the pathway for FeMo-co biosynthesis proceeds normally in the cells (8).

Table 1 shows that NifB is essential for *in vitro* FeMo-co synthesis from its simple constituents. Not adding SAM, Fe, and S to the reaction mixture significantly decreases the NifB-dependent FeMo-co synthesis activity (Table 1). The requirement of SAM for FeMo-co synthesis is evident when desalted

Table 1. Requirements for NifB-dependent *in vitro* synthesis of FeMo-co

Assay	Dinitrogenase activity, nmol of C_2H_4·min^{-1} per assay	
	Crude extract	Desalted extract
Complete	5.7 ± 0.8	2.4 ± 0.2
−NifB	0.1 ± 0.0	ND
−SAM	2.9 ± 1.2	0.5 ± 0.0
−SAM, +0.50 mM SAH	0.5 ± 0.1	0.1 ± 0.0
$-Fe^{2+}$, $-S^{2-}$	2.1 ± 1.7	1.9 ± 0.1
−SAM, $-Fe^{2+}$, $-S^{2-}$	1.1 ± 0.7	0.1 ± 0.0
−NifB, −SAM, $-Fe^{2+}$, $-S^{2-}$	0.1 ± 0.0	ND
Air-oxidized NifB	0.6	ND

Details of the complete assay can be found in *Materials and Methods.* FeMo-co synthesis was determined by acetylene reduction activity of matured dinitrogenase (8). When noted, UW45 cell-free extracts were desalted as described in *Materials and Methods.* Data are the average of two to six independent determinations ± SEM. ND, not determined.

UW45 extracts are used (Table 1). Addition of SAM, Fe^{2+}, S^{2-}, molybdate, and homocitrate as the only low-molecular-weight components to a desalted UW45 extract supports the NifB-dependent FeMo-co synthesis. NifB activity is O_2 sensitive; 30 s of air exposure results in 90% activity lost. Although the presence of SAM stimulates FeMo-co synthesis, addition of the nonreactive analog *S*-adenosylhomocysteine (SAH) inhibits the reaction (Table 1). Excess of SAM in the reaction mix relieves SAH inhibition (Table 2). Finally, addition of 0.5 mM L-cysteine did not substitute for Na_2S (data not shown). Overall, our spectroscopic and kinetic analyses suggest that the as-isolated NifB is not loaded with a FeMo-co precursor (i.e., NifB-co). Rather, these results strongly support a model in which NifB assembles a metal cluster that serves as a FeMo-co precursor upon incubation with Fe, S, and SAM *in vitro*.

We further verify NifB-dependent incorporation of ^{55}Fe from $^{55}FeCl_3$ into apodinitrogenase. NifB-dependent *in vitro* FeMo-co synthesis assays were performed in the presence of $^{55}FeCl_3$. Then, antibodies to dinitrogenase were added to the reaction mixture to immunoprecipitate dinitrogenase. Quantification of radioactivity in the immunoprecipitate clearly shows incorporation of ^{55}Fe from $^{55}FeCl_3$ into apodinitrogenase (Fig. 3). ^{55}Fe-labeled dinitrogenase is active in the acetylene reduction assay. That the ^{55}Fe label is specific for dinitrogenase is demonstrated by the low amount of immunoprecipitated ^{55}Fe material when nonspecific serum is used (2.4×10^5 cpm) and by competition for anti-dinitrogenase antibodies binding when a large excess of nonlabeled pure dinitrogenase is added (Fig. 3). Failure to add NifB to the reaction mix or inhibition of NifB activity by SAH eliminates ^{55}Fe label in the precipitated dinitrogenase and dinitrogenase activity (Fig. 3). Thus, in this *in vitro* assay, ^{55}Fe from $^{55}FeCl_3$ is incorporated into apodinitrogenase when the FeMo-co biosynthesis pathway is active. To determine whether this ^{55}Fe labeling occurs at the level of NifB reaction or downstream in the FeMo-co synthesis pathway, we performed the NifB-co-dependent FeMo-co synthesis reaction with unlabeled NifB-co in the presence of $^{55}FeCl_3$. In this reaction, ^{55}Fe label incorporated into a normalized amount of matured dinitrogenase is only 14% of that of the NifB-dependent reaction (data not shown), strongly suggesting that most of the Fe in FeMo-co originates from NifB-co. It is possible that, in fact, all of the Fe in FeMo-co originates from NifB-co and that the observed 14% of ^{55}Fe label in reconstituted dinitrogenase is due to isotopic exchange during FeMo-co synthesis.

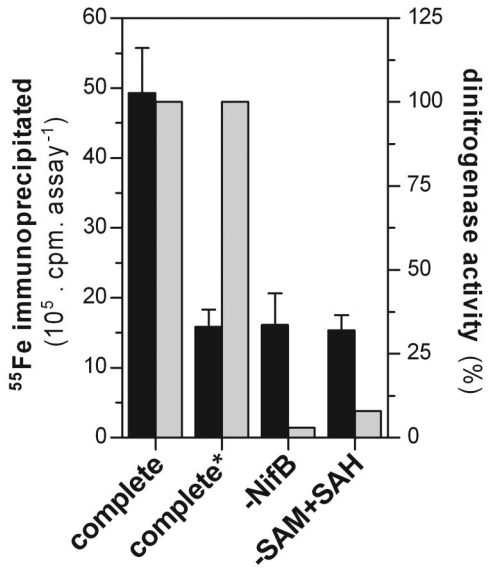

Fig. 3. NifB-dependent incorporation of ^{55}Fe into apodinitrogenase. In the complete assay, labeled dinitrogenase was immunoprecipitated with anti-NifDK antibodies (α-NifDK). As a control, we prepared parallel reaction mixtures (complete*) to which an excess of nonlabeled pure dinitrogenase was added before incubation with α-NifDK beads. The amount of immunoprecipitated ^{55}Fe was quantified by scintillation counting (black bars). Results are the average of two to four determinations ± SEM. Duplicates of reaction mixtures were used to determine dinitrogenase activity by standard procedures (for gray bars, 100% activity corresponds to 4.2 ± 0.1 nmol of ethylene produced per min) (31).

Table 2. Effect of SAM and SAH on NifB-dependent *in vitro* FeMo-co synthesis

Assay	Activity, nmol of C_2H_4·min^{-1} per assay
−SAM	2.9 ± 1.2
+0.10 mM SAM	7.1 ± 0.3
+0.25 mM SAM	7.9
+0.50 mM SAM	7.7
+0.10 mM SAH	2.6 ± 0.2
+0.25 mM SAH	0.7 ± 0.4
+0.50 mM SAH	0.5 ± 0.1
+0.25 mM SAM, +0.10 mM SAH	5.6 ± 0.1
+0.25 mM SAM, +0.25 mM SAH	5.0 ± 0.5
+0.25 mM SAM, +0.50 mM SAH	4.7 ± 0.3

Details of the complete assay can be found in *Materials and Methods.* FeMo-co synthesis was determined by acetylene reduction activity of matured dinitrogenase (8). Data are the average of two independent determinations ± SEM.

Iron–sulfur proteins are common to all organisms. Besides being fundamental components of respiratory and photosynthetic electron transport chains, the iron–sulfur clusters ([Fe–S]) have critical functions in catalysis, redox sensing, protein structure, and radical chemistry (19, 20). The most simple and common [Fe–S] clusters found in proteins are [2Fe–2S] and [4Fe–4S] clusters coordinated by cysteine residues from the polypeptides. The goals of chemical synthesis of simple [Fe–S] clusters have been largely achieved (21). Biochemical synthesis of simple [Fe–S] clusters is currently an active field of research. Almost 40 years have elapsed from the first report of *in vitro* spontaneous assembly of a ferredoxin [Fe–S] cluster (22) to the discovery of complex cellular machineries dedicated to the assembly of [2Fe–2S]- and [4Fe–4S]-containing proteins (19, 23). The enormous complexity of FeMo-co has impaired attempts of chemical synthesis, but very important advances in this

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direction have been made over the last 25 years (24, 25). The development of the NifB-dependent *in vitro* FeMo-co synthesis activity assay reported here, in which purified NifB, SAM, Fe^{2+}, and S^{-2} substitute for NifB-co, links the research of simple and complex metallocluster biosynthesis. Our results strongly suggest that SAM radical chemistry is used for the synthesis of the [Fe–S] cage of FeMo-co. Recent reports implicate the HydE and HydG SAM radical proteins in the assembly of another complex metallocluster, the active site of the Fe-only hydrogenase (26, 27). The elucidation of the role of SAM in [Fe–S] cluster biosynthesis will be the next step in adding to our knowledge of cluster assembly.

Recently, a high-resolution crystal structure of nitrogenase has revealed the presence of a multicoordinated light atom in the center of FeMo-co, which has been proposed to be N, C, or O (4). Further spectroscopic analysis suggests that this central atom might be C but not N (28). Whatever the identity of this atom, it is expected to be in a highly oxidized state, and it is reasonable that very low-potential radical chemistry, carried out by NifB, is responsible for the incorporation of such species into FeMo-co.

Amino acid sequence analysis of NifB indicates that it is composed of at least two different domains: an SAM radical domain at the N-terminal portion of NifB and a NifX-like domain at the C-terminal portion of the polypeptide. The NifX-like domain is found in a family of small proteins able to bind FeMo-co and NifB-co, such as NifX, NafY, and VnfX (11). Because NifB activity generates NifB-co, the role of the C-terminal portion of NifB could be (*i*) a site that binds NifB-co precursors (some type of [Fe–S] cluster) and serves as a scaffold for the formation of NifB-co and/or (*ii*) a site that binds preassembled NifB-co for subsequent delivery to the next protein involved in FeMo-co synthesis, most likely NifNE. The purification of NifB and the development of a NifB activity assay will allow us to study in more detail NifB-co synthesis. At present, we do not know whether both NifB domains are necessary for NifB-co synthesis. Splitting NifB into two domains and analyzing their activities separately could help understand the mechanism of reaction of NifB.

Materials and Methods

Bacterial Growth Conditions. *A. vinelandii* strain UW232 (Δ*nifB*, $nifB_{his}HDK$) was cultivated in a 250-liter fermentor (IF-250; New Brunswick Scientific) in 200-liter batches of modified Burk's medium with limiting ammonium (80 $\mu g \cdot ml^{-1}$ N). Cells were collected by centrifugation 3 h after ammonium exhaustion from the medium. *A. vinelandii* strains DJ (wild type) and UW45 [*nifB* mutant (29)] were cultivated in 20-liter carboys and derepressed for nitrogenase expression as described in ref. 30. Bacterial growth was estimated from the light-scattering of culture samples at 580 nm with a Shimadzu UV-1601 spectrophotometer.

Generation of *A. vinelandii* Strain UW232. The *nifB* gene was shuffled in the chromosome of strain UW232 from its wild-type position to a different position in which it forms a chimeric operon together with the structural genes of nitrogenase (*nifB-HDK*). Full experimental details are provided in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Purification of NifB. All purification steps were performed under an N_2 atmosphere at 8°C. Cell-free extracts from 250 g of cells of *A. vinelandii* strain UW232 were prepared by osmotic shock according to ref. 30 with modifications. NifB was purified from the cell extract by affinity chromatography to a Co^{2+} resin followed by anion exchange chromatography on Q-Sepharose. Cell-free extracts were prepared in buffer A (50 mM sodium phosphate buffer, pH 7.4/300 mM NaCl/20 mM imidazole/10% glycerol/0.05% *n*-dodecyl-β-D-maltopyranoside/5 mM 2-mercaptoethanol/0.5 mM DTH) and loaded onto a 20-ml Co^{2+}-affinity column (Talon resin; Clontech). The column was washed with 5 vol of buffer A and 5 vol of buffer A containing 60 mM imidazole. NifB was eluted with 2 vol of buffer B (50 mM Tris·HCl, pH 8.0/300 mM imidazole/20% glycerol/0.2% *n*-dodecyl-β-D-maltopyranoside/5 mM 2-mercaptoethanol/1.0 mM DTH). The yellowish protein-containing fractions were diluted 1:4 in buffer to adjust them to the composition of buffer C (50 mM Tris·HCl, pH 8.0/75 mM imidazole/5% glycerol/0.05% *n*-dodecyl-β-D-maltopyranoside/5 mM 2-mercaptoethanol/1.0 mM DTH) and loaded onto a 20-ml Q-Sepharose column (Amersham Pharmacia Biosciences) previously equilibrated in buffer C. The column was washed with 5 vol of buffer C supplemented with 180 mM NaCl, and NifB was eluted with 2 vol of buffer D (50 mM Tris·HCl, pH 8.0/400 mM NaCl/20% glycerol/0.2% *n*-dodecyl-β-D-maltopyranoside/5 mM 2-mercaptoethanol/1.0 mM DTH). The yellowish protein-containing fractions were concentrated by ultrafiltration with a 10-kDa cutoff membrane filter (Amicon) and stored under liquid nitrogen in buffer D.

***In Vitro* Reconstitution of NifB [Fe–S] Clusters and UV-Visible Spectroscopy.** Samples of pure NifB protein in buffer D (150 μl) were desalted by passage through 1.8-ml Sephadex G-25 columns (Sigma) equilibrated in buffer E (50 mM Tris·HCl, pH 7.5/150 mM NaCl/10% glycerol/0.1% *n*-dodecyl-β-D-maltopyranoside/5 mM 2-mercaptoethanol). The desalted samples (≈10 μM NifB monomer) were incubated with 10 mM DTT in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) at room temperature for 10 min. Then, 200 μM $(NH_4)_2Fe(SO_4)_2$ and 200 μM Na_2S were added, and the mixture was incubated inside the anaerobic chamber at room temperature for 1 h. NifB samples were desalted again by passage through 1.8-ml Sephadex G-25 columns equilibrated in buffer E. When indicated, 1 mM SAM or 5 mM DTH was added after gel filtration. The UV-visible spectra were determined by using a Shimadzu UV-1601 spectrophotometer.

Desalting of UW45 Cell-Free Extracts. For the removal of low-molecular-weight components from the *A. vinelandii* UW45 cell-free extracts, 2.5-ml aliquots of the extracts were anaerobically desalted by passage through PD10 columns (Amersham Pharmacia Biosciences) equilibrated in 25 mM Tris·HCl (pH 7.5)/10% glycerol/1 mM DTH. Desalted extracts were then concentrated to the initial volume by ultrafiltration in Centricon units with a 3-kDa molecular-mass cutoff (Amicon).

***In Vitro* Synthesis of FeMo-co.** The *in vitro* FeMo-co synthesis reactions were performed in 9-ml serum vials sealed with serum stoppers. The vials were repeatedly evacuated, flushed with argon gas, and rinsed with 0.3 ml of anaerobic buffer. The complete reactions contained 17.5 μM Na_2MoO_4, 0.175 mM homocitrate, 1.75 mM $(NH_4)_2FeSO_4$, 1.75 mM Na_2S, 0.88 mM SAM, 1.23 mM ATP, 18 mM phosphocreatine, 2.2 mM $MgCl_2$, 3 mM DTH, 3.5% glycerol, 46 $\mu g \cdot ml^{-1}$ creatine phosphokinase, 2 μM NifH, 4.7 $mg \cdot ml^{-1}$ UW45 proteins, and 0.48 μM NifB (dimer) in 22 mM Tris·HCl (pH 7.5). The reactions (total volume of 550 μl) were incubated at 30°C for 35 min to allow for the FeMo-co synthesis and insertion reactions. The resulting activation of apodinitrogenase present in UW45 extract was analyzed by the acetylene reduction assay following standard procedures (31). NifB-co-dependent *in vitro* synthesis of FeMo-co assays was performed as described in ref. 8. Briefly, NifB-co was extracted from *Klebsiella pneumoniae* strain UN1217 [*nifN*::mu insertion mutant (32)] cytoplasmic membranes solubilized with 1% *n*-lauroyl-sarcosine and purified by Sephacryl S-200 and Phenyl-Sepharose chromatographies. NifB-co preparations (≈0.5 mM in Fe content) were stored in

liquid nitrogen in 50 mM Mops buffer (pH 7.5)/1 mM DTT/2% *n*-lauroyl-sarcosine. In time-course experiments of NifB-dependent and NifB-co-dependent FeMo-co synthesis, reactions were stopped at different times by addition of 0.3 mM $(NH_4)_2MoS_4$ as described in ref. 33. Acetylene reduction activity of matured dinitrogenase was then determined by standard procedures (31).

^{55}Fe Labeling and Immunoprecipitation of NifDK. For ^{55}Fe labeling experiments, a modified version of the complete NifB-dependent FeMo-co synthesis assay (above) was developed. Reaction mixtures contained 28 μM $^{55}FeCl_3$ [0.45 mCi·ml^{-1} (1 Ci = 37 GBq)] and 250 μM Na_2S in 58 mM Tris·HCl (pH 7.5). The concentration of all other components remained unchanged. The reactions (total volume of 1.1 ml) were incubated at 30°C for 35 min to allow for the FeMo-co synthesis and insertion reactions. The reaction mixtures were then incubated with protein A-Sepharose beads containing either anti-NifDK (α-NifDK) or preimmune serum IgG at 2 mg of IgG per ml of wet beads prepared as in ref. 34. Immunoprecipitation and quantification of ^{55}Fe bound to the protein A-Sepharose beads were performed as described in ref. 34. A specificity control was performed by adding a large excess of unlabeled purified dinitrogenase (1.2 mg of NifDK) before immunoprecipitation to compete for the α-NifDK antibodies.

Miscellaneous Methods. Protocols and references to *in vivo* and *in vitro* nitrogenase activities, protein determination assays, SDS/PAGE, immunoblot analysis, anoxic native PAGE, iron staining of native gels, iron determination in protein preparations, and analytical gel filtration are provided in *Supporting Materials and Methods*.

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